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(54) Nuclear transplantation

(57) A process for nuclear transplantation wherein a donor nucleus and a recipient cytoplast are fused in suspension in an aqueous medium under conditions wherein the medium contains substantially no extracellular free calcium ions, whereby the fusion occurs without inducing activation of the resultant reconstituted embryo, is described.

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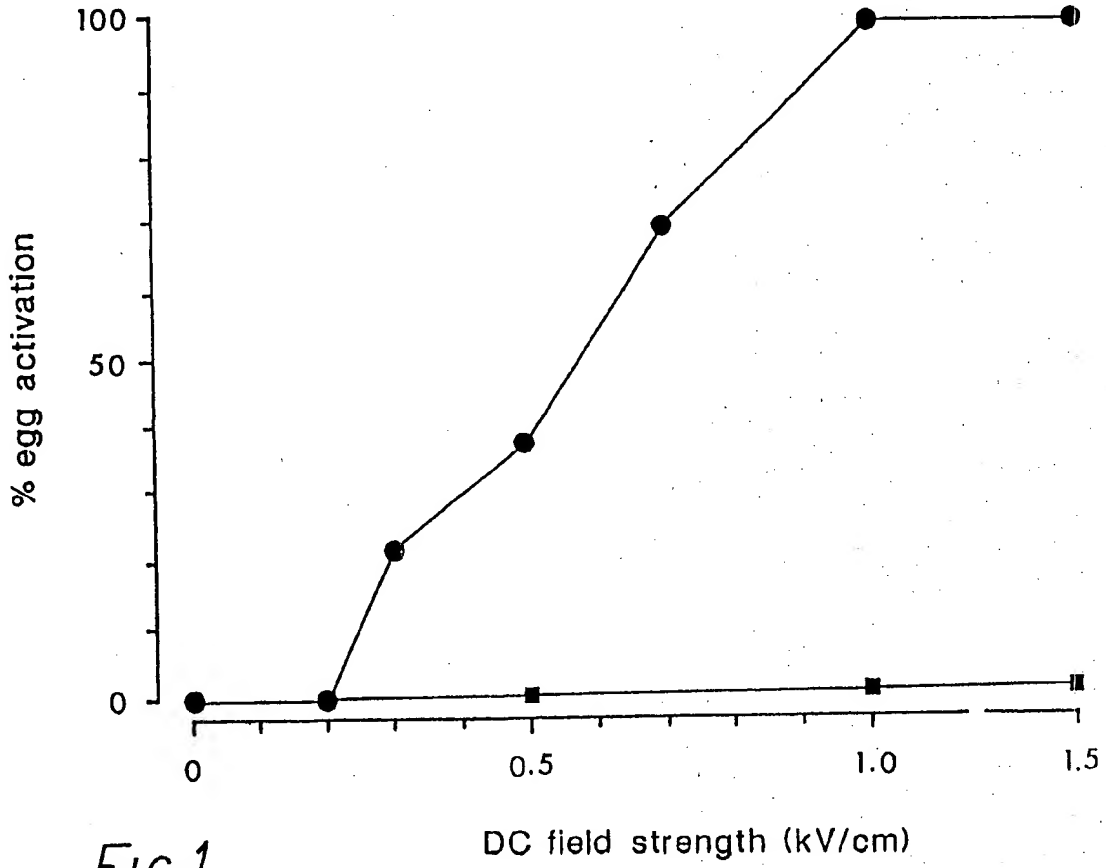


FIG. 1

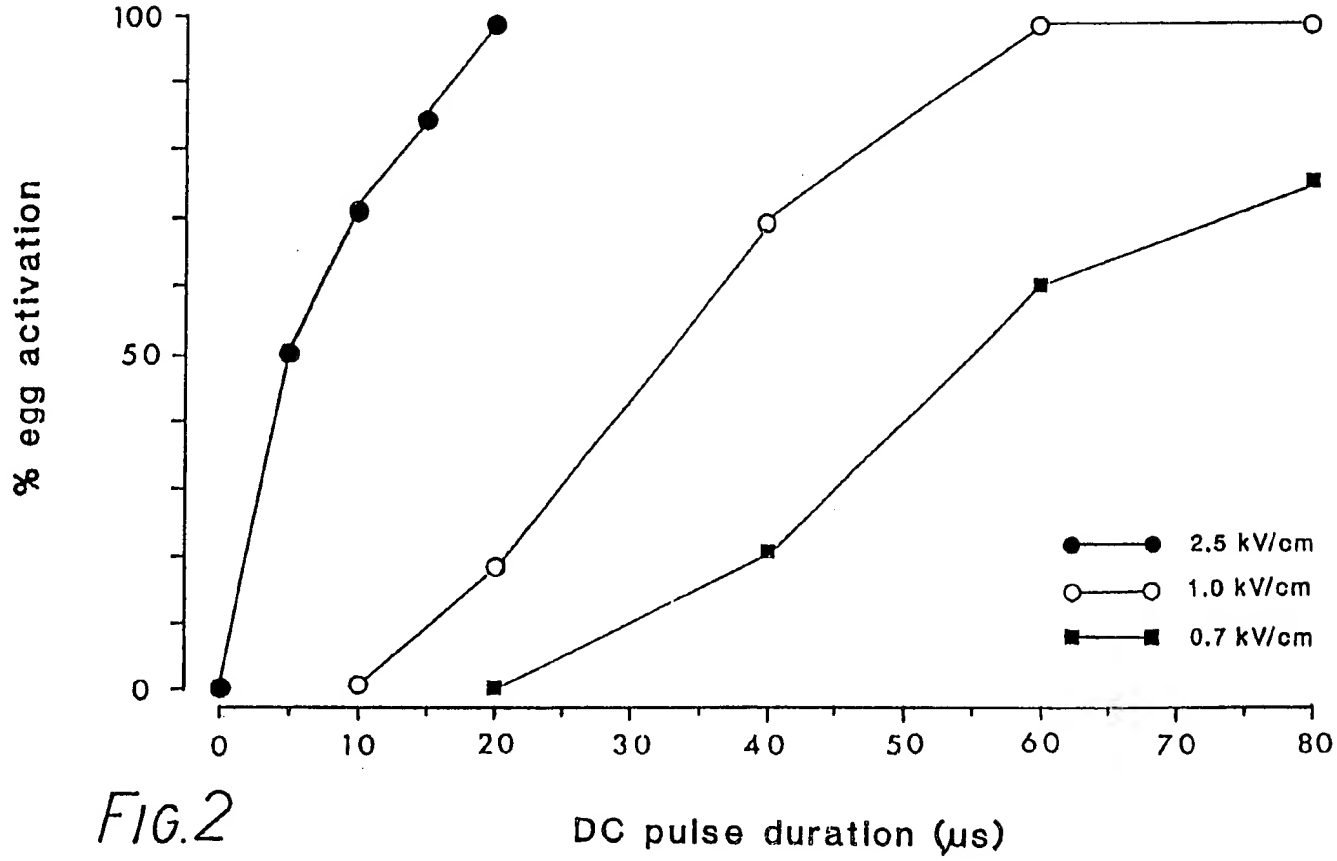

100
50
0
% egg activation
0
10
20
30
40
50
60
70
80
DC pulse duration (μs)
2.5 kV/cm
1.0 kV/cm
0.7 kV/cm

FIG.2

FIG. 3

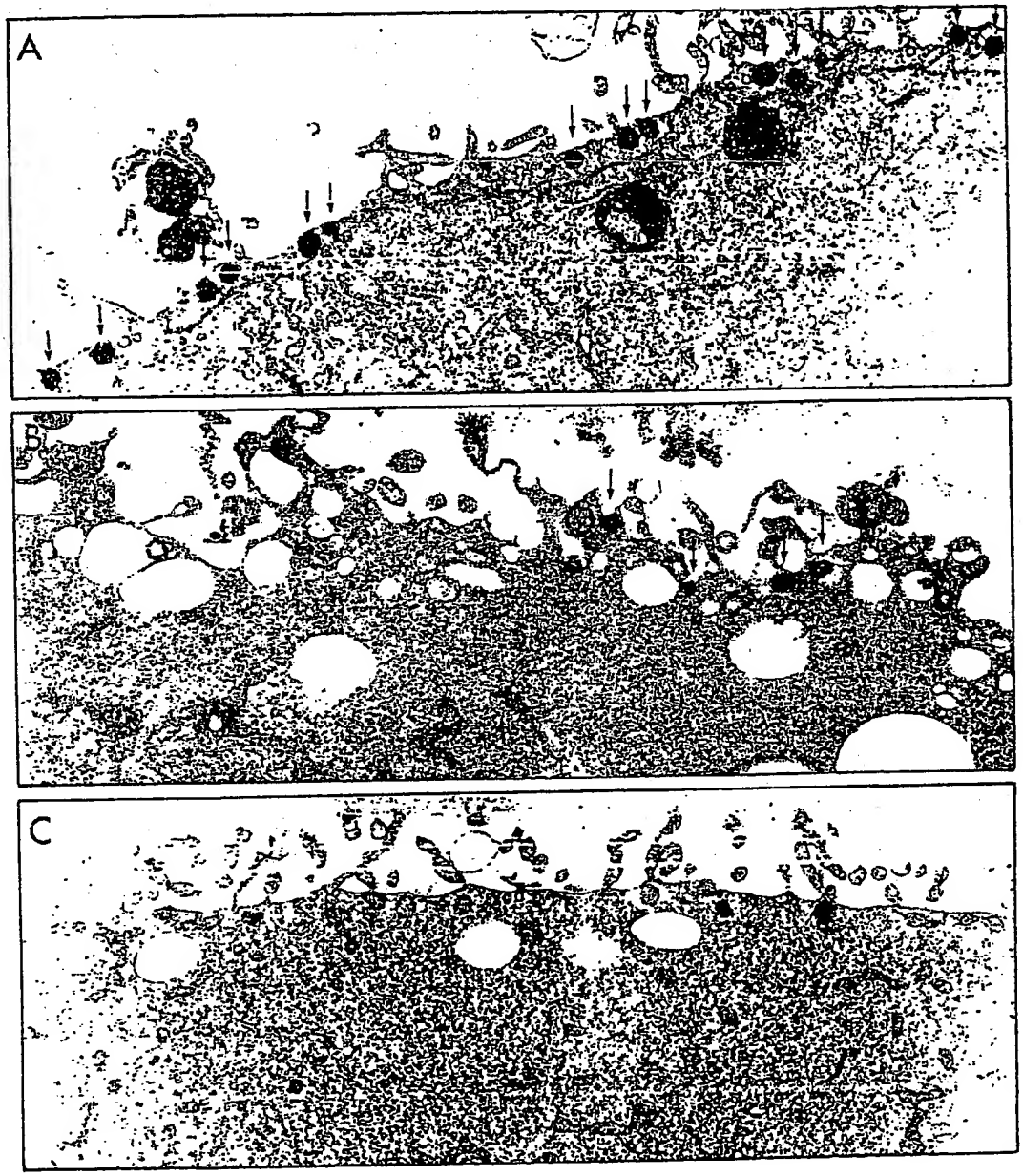


FIG.4

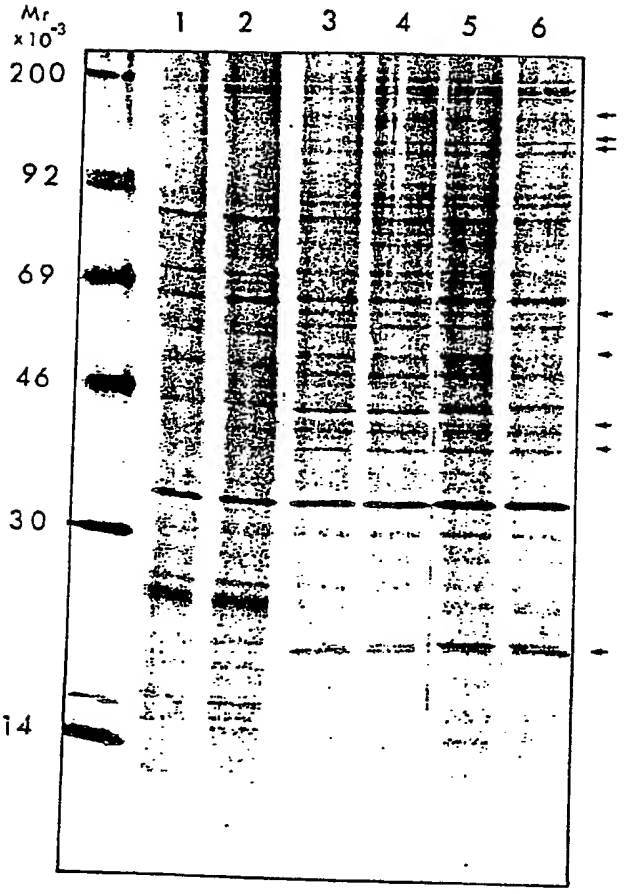


FIG. 5

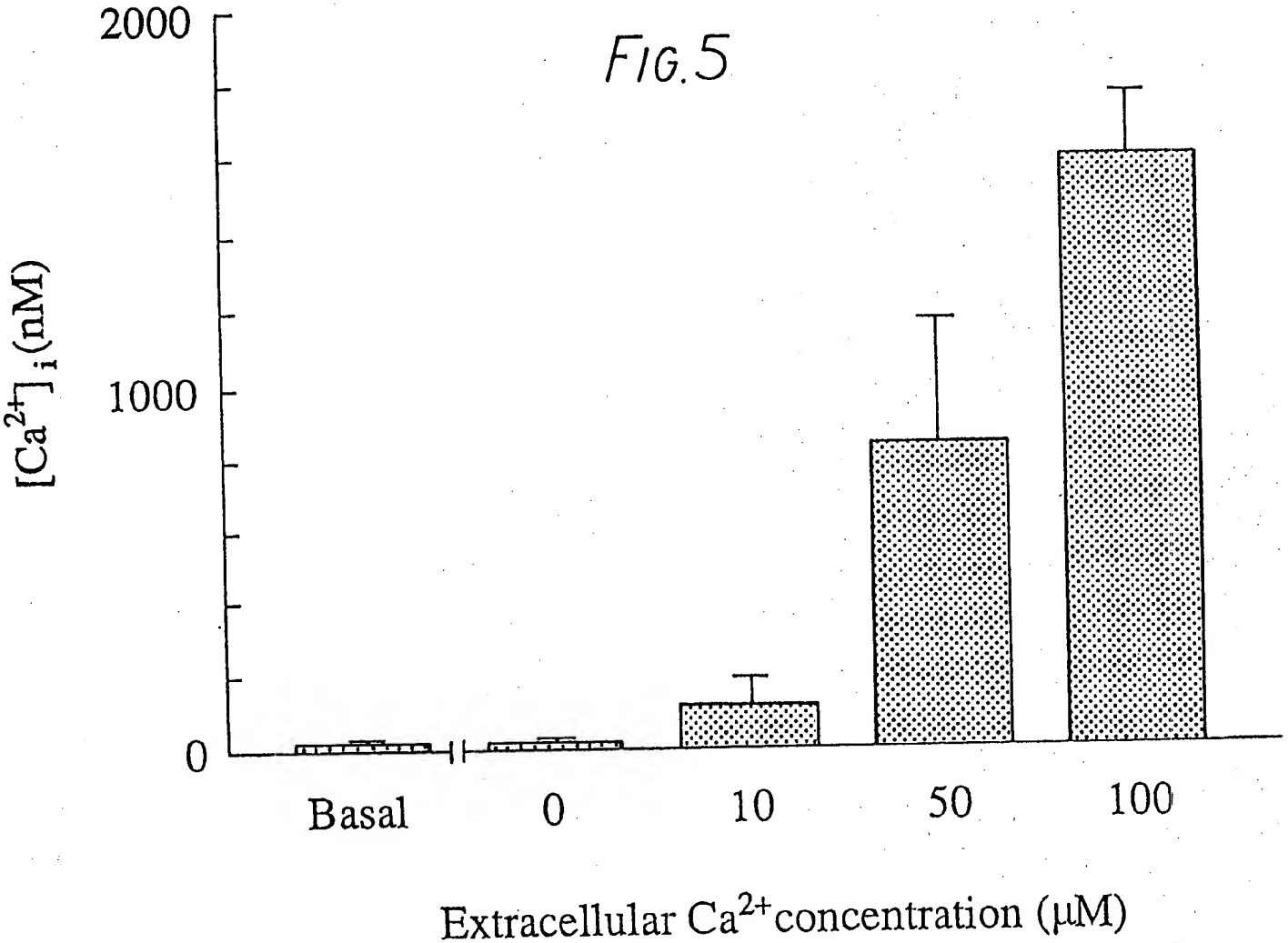

2000
1000
0
$[Ca^{2+}]_i$ (nM)
Basal
0
10
50
100
Extracellular Ca^{2+} concentration (μM)

FIG. 6

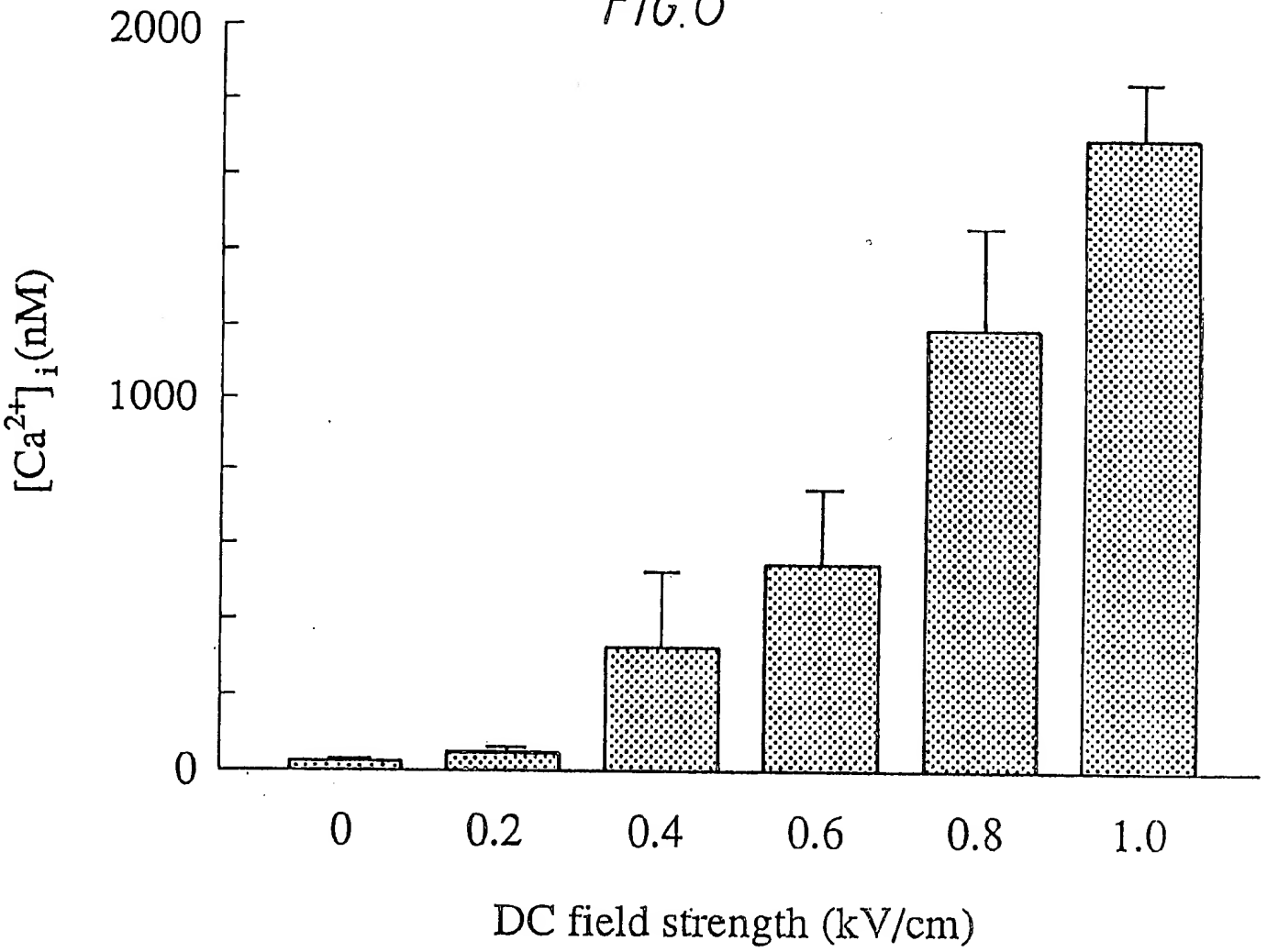


FIG. 7

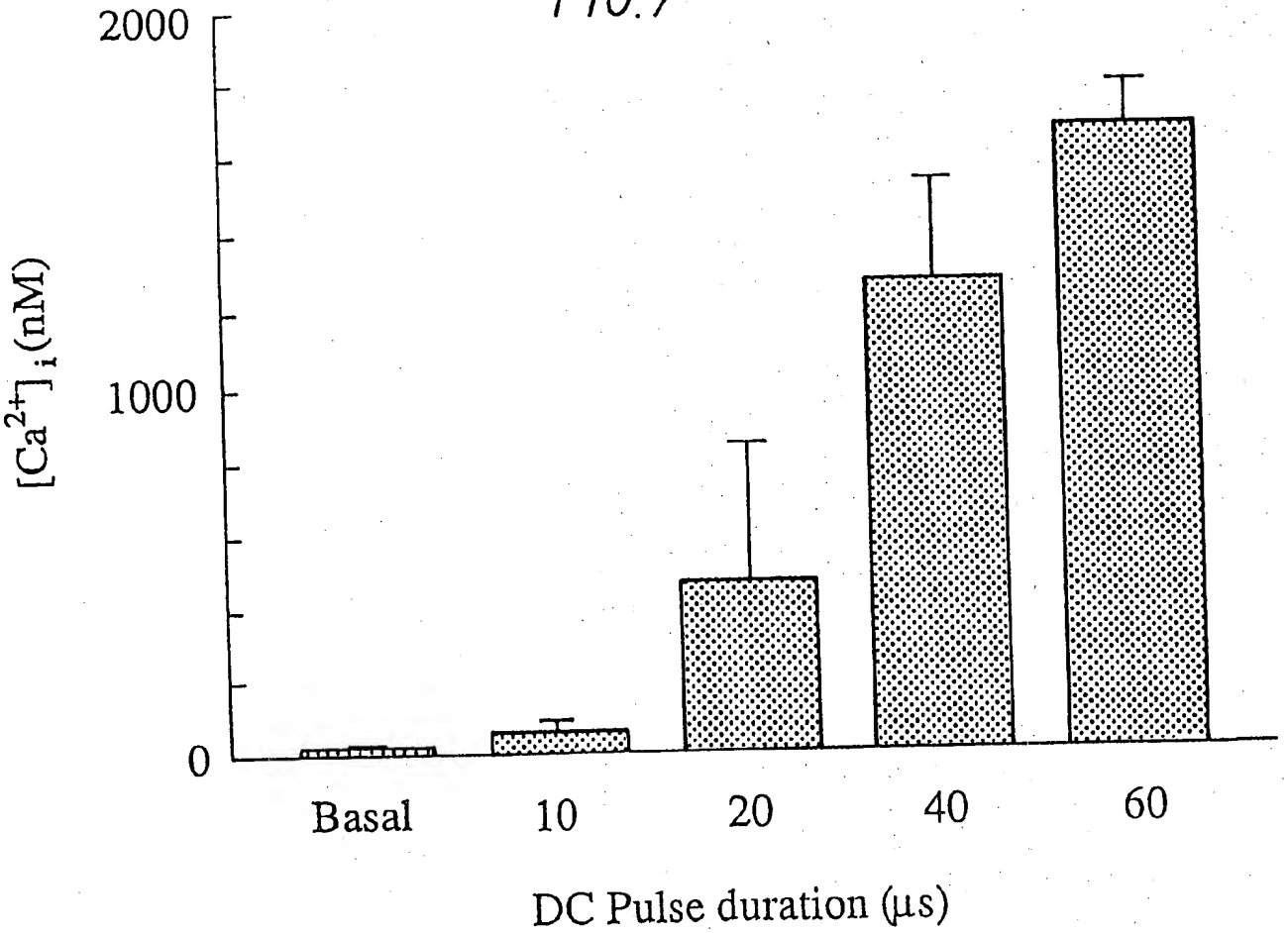


FIG. 8

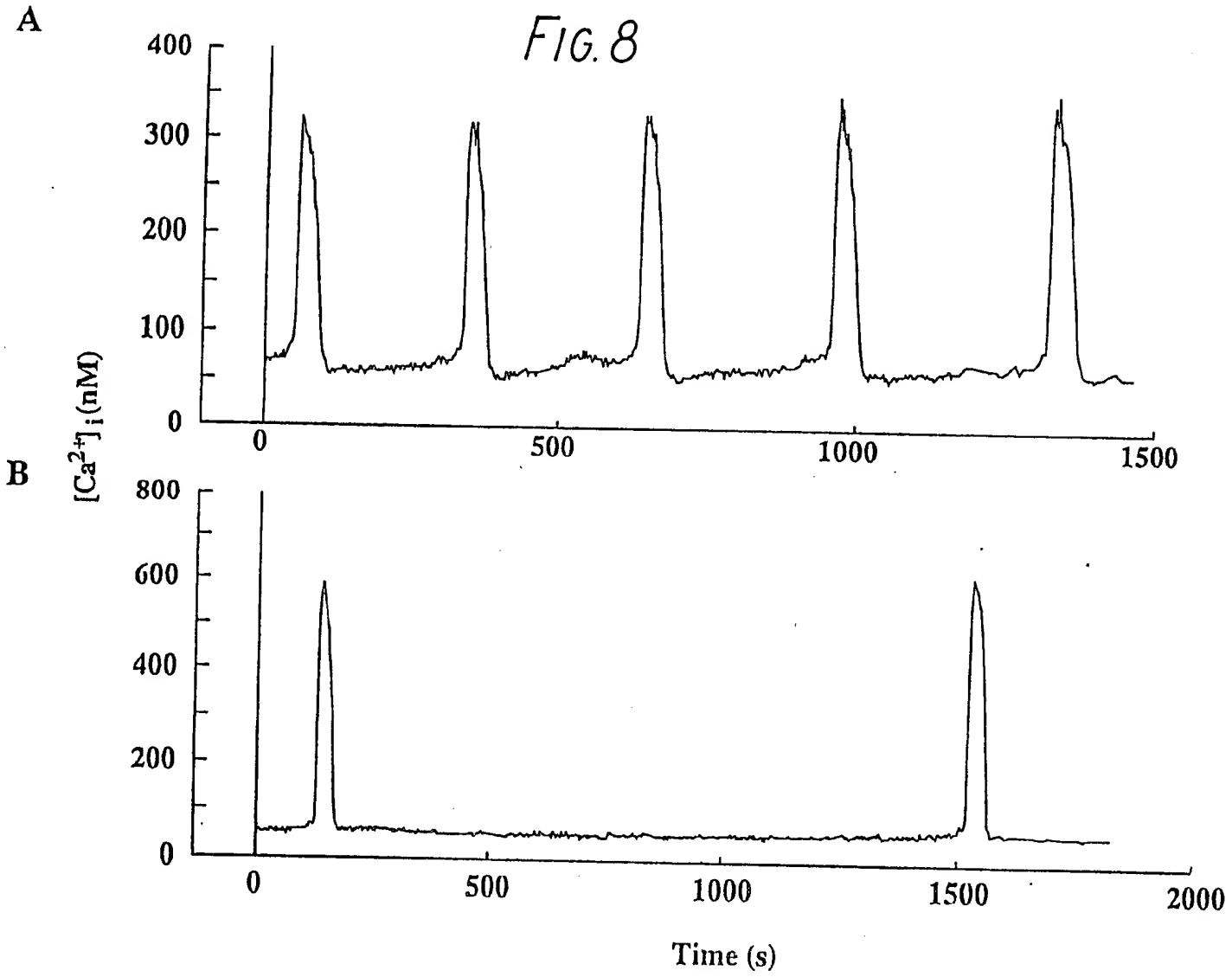

A
B
400
300
200
100
0
800
600
400
200
0
0
500
1000
1500
2000
$[Ca^{2+}]_i$(nM)
Time (s)

FIG. 9

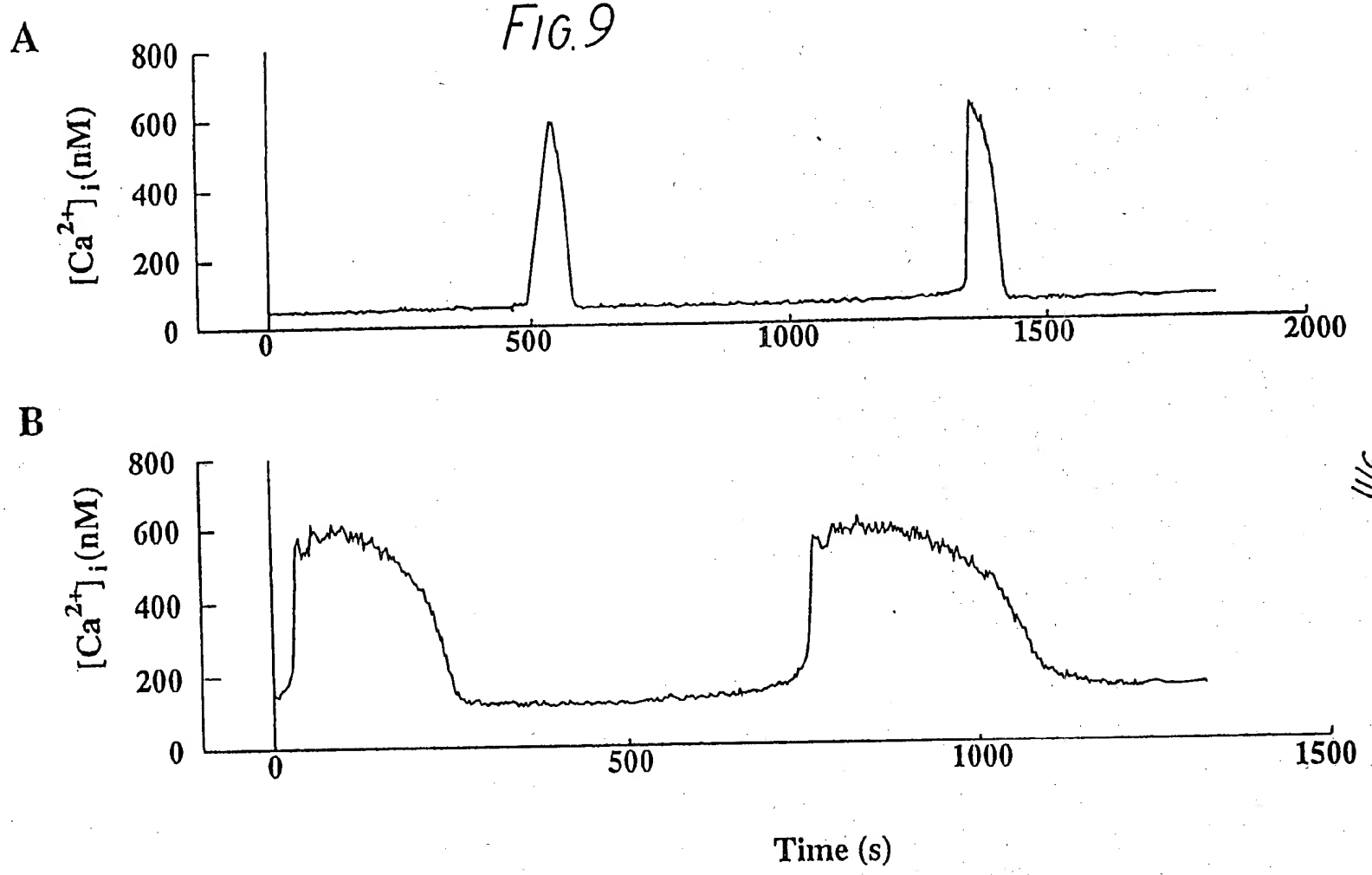
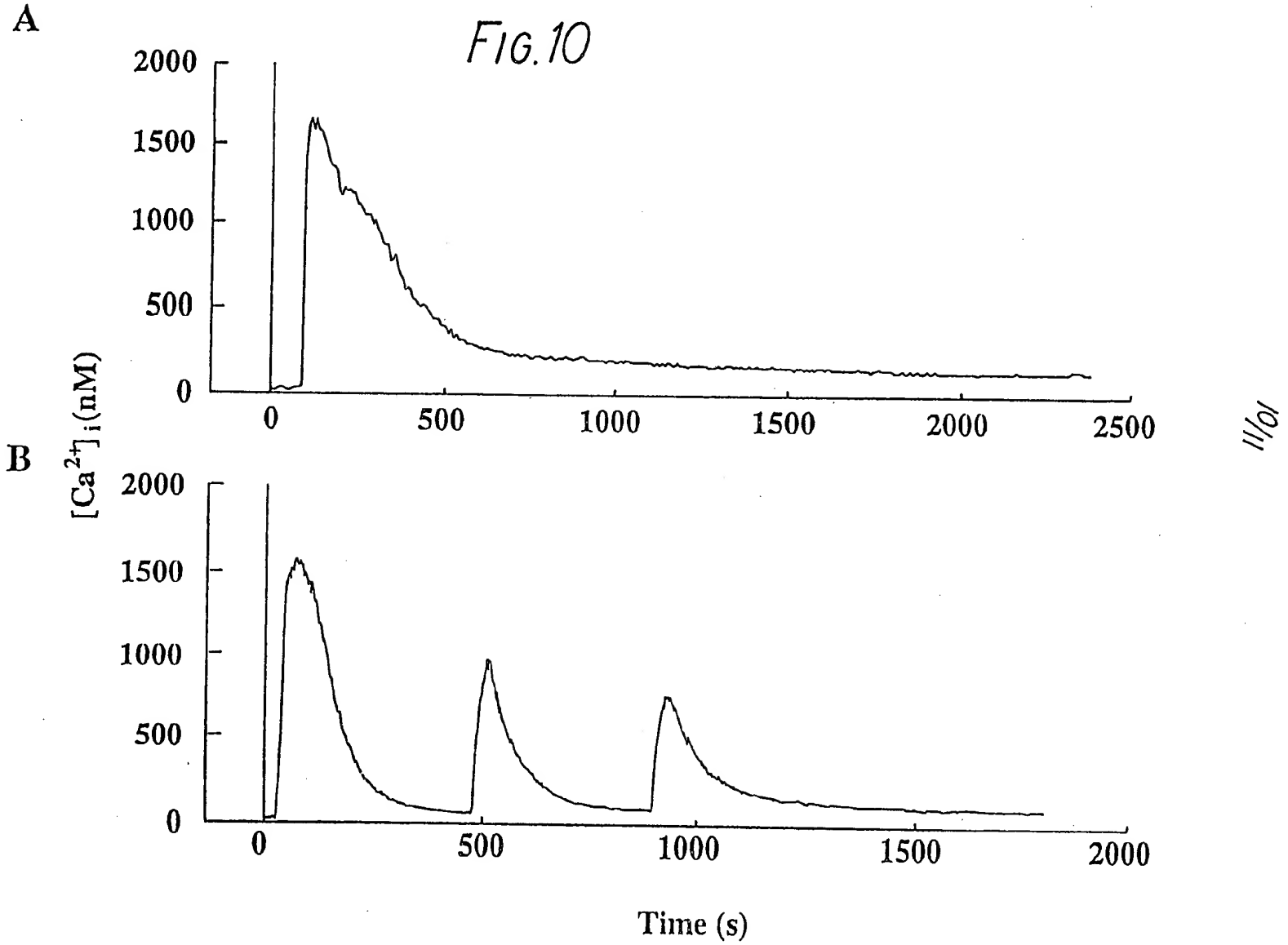

A
800
600
400
200
0
$[Ca^{2+}]_i$(nM)
0
500
1000
1500
2000
B
800
600
400
200
0
$[Ca^{2+}]_i$(nM)
0
500
1000
1500
Time (s)

FIG. 10



11/01

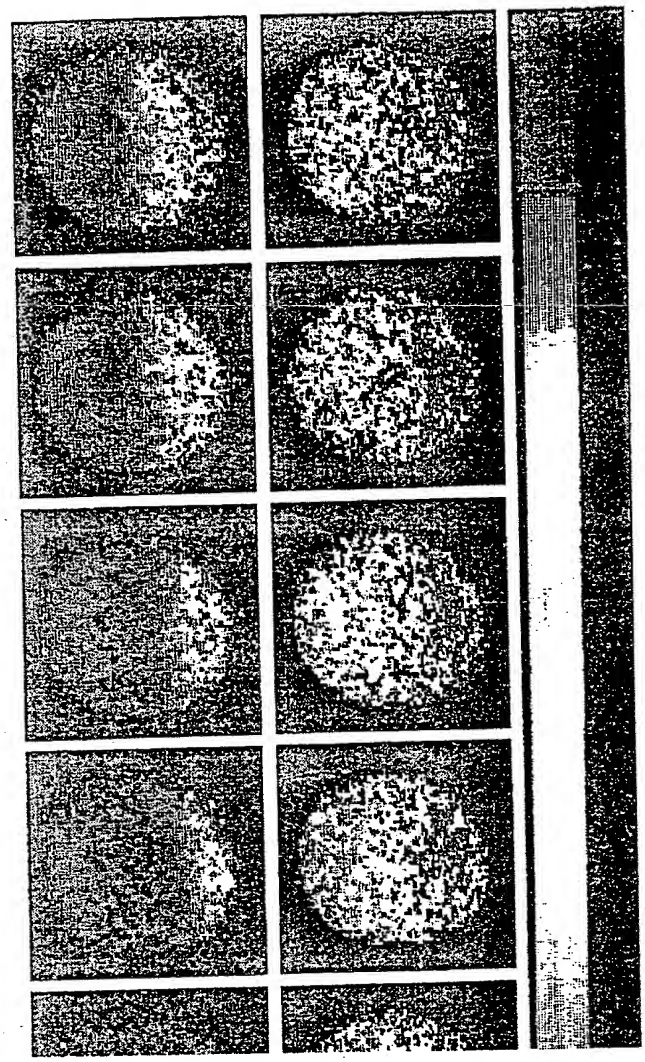


FIG.11

Nuclear Transplantation

Animal cloning by nuclear transplantation has been recognised as a potentially revolutionary biotechnology in improving both the efficiency of animal production and the quality of animal products. It is also believed that if cloning animals by using embryonic stem cells as donor nuclei becomes feasible, nuclear transplantation will undoubtedly be the most efficient and powerful method in producing large numbers of identical transgenic animals.

Generally, nuclear transfer consists of three essential steps, namely, (1) the preparation of recipient cytoplasts (in which the transplanted donor nucleus is expected to undergo reprogramming and gain its ability for directing further development), (2) the preparation of donor nuclei (the nucleus contains the genetic information which will determine the characteristic of the nuclear-transplanted animal), and (3) the nuclear insertion procedure itself (this step leads to the production of a reconstituted egg or embryo). In practice, recipient cytoplasts are produced by removing the metaphase chromosomes of the unfertilized eggs with a fine micropipette; donor cells are obtained from embryos at various stages of development; and nuclear insertion can be achieved by fusing the donor cell and the recipient cytoplast, e.g. by electrofusion.

The basic nuclear transplantation procedures have already been established in most of the domestic animal species (including sheep, cattle and pig). Although a small number of farm animals can be cloned from one donor embryo, it is widely found that the percentage of the reconstituted eggs that can develop to newborn animals is very low. Therefore, identifying the factors that influence the developmental capacity of the nuclear transplanted eggs and establishing the optimal experimental and biological conditions for normal embryo development are the central objective of nuclear transplantation studies.

From our previous studies, we find that egg activation following fusion is the most important single factor determining the further development of the nuclear-transplanted eggs. After nuclear insertion only those reconstituted eggs which have been appropriately activated are able to undergo further cleavage while those that are not activated remain at the one-cell stage until degeneration. We conclude that the full activation of the egg, followed by its continuous stimulation through the induction of multiple calcium transients, are the crucial triggers that initiate development. Based on these conclusions we have extended the investigations by using porcine eggs to define the conditions required for a high rate of activation and ensuing stimulation. The ultrastructural and biochemical changes induced by these treatments have been analysed. This investigation, using electrofusion to achieve fusion of nucleus and cytoplast, has lead to the following observations:

1. High rate of egg activation is the product of an inverse relationship between DC (direct current) field strength and pulse duration. We find that under given conditions 100% of eggs can be activated and that the treated eggs are able to undergo a series of ultrastructural and biochemical changes which are similar to those induced by sperm during fertilization.

2. Dynamic video imaging studies using Fura-2 as Ca^{2+} probe show that electrical stimulation is able to cause a transient increase in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) immediately following the treatment. The transient increase in $[Ca^{2+}]_i$ following stimulation is the direct trigger that induces the initial biological events associated with activation; these changes are initiated only after the intracellular free calcium concentration reaches a defined threshold level.

3. The increase of intracellular free calcium following stimulation is determined both by the DC field parameters and the extracellular Ca^{2+} concentration. Our results suggest that this

electrically-induced Ca^{2+} transient increase is caused by an entry of extracellular Ca^{2+}. In the absence of extracellular calcium eggs show no increase in their intracellular free Ca^{2+} concentration following treatment despite being exposed to adequate stimulation conditions.

4. Our results show that standard electrofusion conditions generally result in the immediate activation of fused oocytes but fail to elicit the post-activation calcium transients normally associated with fertilization. Multiplie transients can however, be induced by stimulating electrially-activated eggs under appropriate conditions with chemical activators or repeated DC-pulses.

Based on the observations described above we have developed a new concept of nuclear transplantation. Our technique involves two distinct biological phases. In the first phase, nuclei are inserted into cytoplasts without inducing egg activation. Our results show that 85% of successful fusions can be carried out without inducing activation of newly reconstituted eggs. After an appropriate period of nuclear-cytoplasmic "conditioning", activation and post-activation Ca^{2+} transients are induced. This new technique is in direct contrast to prior methods of nuclear transplantation in mammalian embryos where insertion of the nucleus inevitably leads to activation and hence fusion and activation of the cytoplasm occur simultaneously.

We consider that the following points are of paramount importance for nuclear transplantation and subsequent development.

1. The development of nuclear-transplanted eggs depends heavily on the ability to induce both initial activation and subsequent egg stimulation by Ca^{2+} transient induction.

Optimal conditions for egg activation have been defined and methods of inducing post-activation calcium transients have been

2. The ability to condition transplanted nuclei in an appropriate cytoplasmic environment before activation is of extreme importance in nuclear reprogramming and in the consequent induction of normal development.

Our method of inducing fusion without activation provides the basis for this new concept in nuclear transplantation. Karyoplasts from a variety of cell types can be fused into cytoplasts at different cell cycle stages. The optimal time for genomic reprogramming or nuclear conditioning in the resultant reconstituted egg combinations can be determined.

The sequential processes of nuclear conditioning followed by optimal activation and calcium stimulation substantially enhances embryonic development after nuclear transplanation. The methods of inducing each of these processes are practical and fully compatible with the requirements for commercial exploitation.

In summary the present invention can (i) provide a new procedure for nuclear conditioning or reprogramming before activation and development (ii) define optimal conditions for egg activation and (iii) identify methods of inducing post-activation calcium transients in reconstituted eggs.

Accordingly, the invention provides a process for nuclear transplantation comprising the steps of:

a) preparing a recipient cytoplast wherein the cytoplast is from an oocyte at the metaphase II stage of meiosis (MII oocyte);
b) preparing a donor nucleus; and
c) fusing the nucleus and cytoplast to produce a reconstituted embryo;

the process being carried out <u>in vitro</u> in a suspension medium;

characterised in that the suspension medium is a physiologically acceptable aqueous solution which contains substantially no free calcium ions. In functional terms, the invention provides a process for nuclear transplantation wherein a donor nucleus and a recepient cytoplast are fused in suspension in an aqueous medium under conditions wherein the medium contains substantially no extracellular free calcium ions, whereby the fusion occurs without inducing activation of the resultant reconstituted embryo.

Eggs which are at a very early stage of development (e.g. MI phase) would not normally be expected to be activated by the fusion process. A problem of immediate activation being caused by the fusion process occurs in cases where the oocyte is relatively more developed (e.g. at the MII phase). Thus, this invention is directed to using oocytes which would, but for the invention, be generally expected to undergo fusion and activation simultaneously.

Hence, the invention further provides a process as described above for fusion of nucleus and cytoplast without causing activation with the additional steps of maintaining the resultant reconstituted embryo without activation for a selected period of time so as to allow nuclear-cytoplasmic conditioning to occur in the intracellular environment, and; stimulating the reconstituted embryo to cause activation of developmental processes.

It is preferred that the suspension medium be devoid of free calcium ions. However, it is to be understood that a certain amount of free calcium could be tolerated (e.g. as a contaminant or impurity or residual trace) in the medium and the invention could still be achieved. For this reason the term "substantially no free calcium ions" may be construed as including the concept "an amount of free calcium ions less than that sufficient to permit activation to occur". The absolute threshold upper limit of permissible calcium ions in the suspension medium is dependent not only on the fusion technique and conditions but also on the oocytes themselves:

however it is presently considered that at a concentration of less than 10μM activation on fusion will generally not occur and at 10μM or above activation will occur. It is preferred to have the least possible amount of free calcium ions in order to ensure no activation (i.e. 5μM or less) and it is most preferred to use calcium free medium.

By way of example, the suspension medium could be about 0.3M Mannitol and about 100μMMg^{2+} in double distilled water at a pH of about 6.8 to 7.0. The same medium could be used in an activation step with the addition of about 100μM calcium.

Techniques and apparatus for electrofusion to achieve nuclear transplantation are already known; however all prior electrofusion techniques employed a significant free calcium concentration in the medium. Electrofusion is the preferred method of carrying out the process of the present invention, and preferably it is achieved using a pulse which provides an electric field of about 0.5-2.0 KV/cm preferably 1.0-1.5 KV/cm in the time period range 0.10-100μs preferably 10-80μs.

However, the invention is not limited to such a technique and could equally be applied using a technique in which fusion is carried out by the use of Sendai virus or in which fusion is carried out by the use of an added solution of polyethylene glycol.

It is clear that the period of time during which the reconstituted egg is maintained without activation will vary depending on many circumstances including the original stage of development of the cytoplast, the time required for the desired level of nuclear reprogramming in the new cytoplasmic environment, the species of donor animal and so on. Theoretically the quiescent period could be of indefinite length, but for the fact that the eggs will "age" due to continued metabolic processes. Thus despite the fact that "development" as such does not occur until activation, the quiescent period has an upper limit. It is considered that

according to this invention the period would normally be about 2-24 hours, and several days at a maximum. It is to be appreciated that a cryogenic storage step could be introduced during that period, which would effectively halt metabolism and aging and could allow fused, unactivated oocytes to be stored indefinitely for thawing and activation at a later date.

Although it is preferred to cause activation by electrostimulation, it is within the scope of the invention to use other agents for this purpose. For example in certain circumstances it may be appropriate that activation is carried out by stimulation with a chemical activator, e.g. one selected from Ethanol, Thimerosol and IP3. Alternatively it may be that activation is carried out by a biological activator such as sperm. Since it is not likely that it will be desired to incorporate any sperm DNA in the oocytes it is preferred that the sperm has been treated by irradiation so as to inactivate the sperm DNA.

Activation by sperm causes multiple transient intracellular calcium concentration changes of characteristic size and duration which are difficult to mimic exactly: however electrostimulation with multiple transient electric pulses can produce intracellular calcium ion transient increases which approximate to those produced by sperm during fertilisation of an embryo, and consequently multiple pulse stimulation is preferred over single pulse electrostimulation.

The invention will now be exemplified with reference to a comparative study of intracellular changes in porcine eggs after fertilisation and electrostimulation. This particularly sets out optimal conditions for stimulation to cause activation which are applicable to nuclear transplanted oocytes made according to the invention. Whilst the examples refer to the use of porcine eggs, the use of eggs from other species (particularly mammalian species, e.g. cattle, sheep and other ungulates) is not excluded.

At fertilization the sperm not only delivers its DNA to the egg but also initiates a developmental programme of physiological, morphological and biochemical changes which launches the dormant egg on a path leading to DNA synthesis and cleavage (Miyazaki, 1990; Whitaker & Patel, 1990). The importance of the genetic contribution of sperm has been well demonstrated by experiments in which it is shown that the paternal and maternal genomes have complementary roles during embryogenesis and both are required for development to term (Surani *et al.*, 1984; McGrath & Solter, 1984). Although cytoplasmic changes associated with egg activation have been less well studied, it is apparent that transient cytoplasmic signals generated at that time markedly alter the function of nucleus (Czolowska *et al.*, 1984; Szollosi *et al.*, 1986, 1988; Sun, 1989).

In recent years electrofusion has become the most widely used method of transplanting nuclei from embryonic cells into enucleated eggs. Since the electrical parameters required for cell fusion are also those able to induce parthenogenetic development it has become standard practice to activate the cytoplast and transplant the nucleus using the same electrostimulus. However, it is unclear whether electrical activation mimics in all respects that induced by sperm penetration. It is apparent that if this were not to be the case, results from electrofusion studies would require re-evaluation.

The comparison of the two systems of activation is a first step in determining the true developmental capacity of embryonic and somatic nuclei by nuclear transplantation. More specifically experiments have been carried out to define the changes that occur in ultrastructure, protein synthesis and ionic balances in porcine eggs following normal fertilization and electro-activation.

MATERIALS AND METHODS

Preparation of porcine oocytes

Oocyte collection: Pig ovaries were obtained from a local abattoir. Intact, non-atretic follicles (>4mm in diameter) were dissected from the ovaries and then opened to remove the entire cumulus-oocyte complex. All these procedures were performed in PBS at room temperature.

Oocyte maturation *in vitro*: Maturation of groups of 20-30 cumulus-enclosed oocytes were carried out in 35 mm plastic culture dishes (Sterilin, UK) containing 2ml of culture medium (TCM 199) supplemented with 10% fetal calf serum (FCS: Sera-Lab Ltd), and gonadotrophins (NIH-LH-022, 2.5μg/ml; NIH-FSH-P2, 2.5μg/ml). In all instances, two freshly prepared follicle shells were added to the medium in which the oocytes were cultured. The maturation was performed at 38.5°C in 5% CO_2 in air.

Egg selection: After 45h of maturation culture, the oocytes were denuded of associated somatic cells. Those oocytes which had extruded their first polar body were identified, isolated and maintained at 38.5°C in TCM 199 + 0.25 mM Hepes supplemented with 10% FCS pending further treatment.

Electrical stimulation and egg culture *in vitro*

The electrofusion system used in this investigation was the same as that described by Sun and Moor (1989, 1991). The basic pulsing medium was 0.3 M mannitol (Sigma) supplemented with 100μM Ca^{2+}, 100μM Mg^{2+} and 0.01% polyvinyl alcohol (PVA). The eggs, after 3 washes in a pulsing medium, were transferred to a fusion chamber (Sun and Moor, 1989) containing the same pulsing medium and stimulated thereafter by a DC pulse treatment using different fields and a variety of pulse durations. After pulse treatment, eggs

were washed in TCM 199 supplemented with 0.25 mM Hepes and 10% FCS and cultured in TCM 199 + 10% FCS at 38.5°C in 5% CO2 in air.

Cytological assessment

After 15h of culture, one group of electrically stimulated eggs were fixed for 48h in ethanol/acetic acid (3:1), stained with lacmoid and assessed for egg activation using phase contrast microscopy.

To assess cortical granule exocytosis following stimulation, a second group of treated eggs were fixed and prepared as described by (Cran & Cheng, 1986) and then examined by transmission electron microscopy.

In vitro fertilization procedures

Fertilization of porcine eggs *in vitro* was performed following the procedures of Cheng (1985). Briefly, sperm-rich fractions (20 ml) were collected from mature boars, and after filtration the sperm were held at 20°C for 16h. Thereafter, 1 ml of semen was washed three times by centrifugation (500g) for three times in PBS supplemented with BSA (3mg/ml) to remove seminal plasma. After the final wash, the sperm were resuspended at 4×10^8 cells/ml in a preincubation medium consisting of TCM199 + 0.25 mM Hepes Sodium pyruvate, 3.05 mM D-glucose and antibiotics. The sperm suspension was preincubated for 90 min at 38.5°C under 5% CO_2 in air. After that, aliquots of the preincubated sperm were added to the fertilization medium consisting of TCM199 supplemented with the same additives as the preincubation medium. The final sperm concentration in the medium was 1×10^5 cells/ml.

Radiolabelling and electrophoretic analysis of labelled proteins in eggs and zygotes

Comparisons were made of the pattern of proteins synthesised by control eggs and eggs after activation by sperm penetration or electrostimulation. Fertilised eggs at 8h post insemination were transferred to BOCM-2 medium (Cheng, 1985) and cultured for a further 12h before radiolabelling. Groups of 10 to 15 denuded eggs or zygotes were labelled at 38.5°C for 3h in 50 μl of labelling medium (Moor et al. 1980) containing [^{35}S]methionine (1000 Cimmol^{-1}, Amersham) at a radioactive concentration of 500 μCi/ml. After labelling, the eggs and zygotes were washed in 10mM Tris-HCl, pH 7.4, and transferred to plastic tubes in a minimal volume of the Tris-buffer (<2μl), and then lyophilized and frozen at -70°C until required for electrophoresis.

Radiolabelled proteins in the eggs and zygotes were analysed on one-dimensional gels as described by Sun and Moor (1991). The scanning of the gels and quantitative analysis of protein bands were carried out by using a Chromoscan 3 densitometer (Joyce-Loebl, England).

Measurement of intracellular calcium

Changes in $[Ca^{2+}]_i$ were measured in (1) control eggs, (2) in electrically activated eggs and (3) in eggs activated by sperm penetration. In all groups, newly matured eggs were incubated at 38.5° with the calcium sensitive fluorescent dye, fura-2 acetoxymethyl ester (fura-2/AM; 4μM) for 45 min in TCM 199 supplemented with 0.25 mM Hepes and 10% FCS. After that, they were washed five times in culture medium and then maintained in culture at 38.5°C pending further treatments. For measuring $[Ca^{2}]_i$ change after electroactivation, fura-2/AM loading eggs were extensively washed in a pulsing medium before being placed in a modified Krüss stimulation chamber (Krüss, Hamburg, Germany).

The modifications involved replacing the supporting glass in the chamber with a thin glass coverslip (22 mm diameter, ARH, England) and introducing a culture well (Sun & Moor, 1989) into the chamber. This modified chamber enabled eggs to be electroactivated and their $[Ca^{2+}]_i$ changes monitored under the same set of conditions. For the analysis of $[Ca^{2+}]_i$ change after sperm penetration, the fura-2/AM prelabelled eggs were briefly exposed to prewarmed acid tyrode solution for zona removal (Nicholson *et al.*, 1975) and then extensively washed before transfer either to normal fertilisation medium or to the fertilisation chamber which consisted of a thin coverslip (22 mm diameter, ARH) fitted into a stainless steel well which was maintained at 38.5° by a thermostatically controlled heating block. The volume of fertilisation medium was typically 300 μl covered by light liquid paraffin (BDH, UK).

Dynamic video imaging was performed using the Magiscan hardware and TARDIS software supplied by Joyce Loebl (Dukesway, Team Valley, Gateshead, UK) following the procedures as described by Neylon *et al* (1990). All the fluorescent measurements were performed on an inverted Nikon Diaphot epifluorescence microscope. Ratios (340nm/380nm) of 0·30, 1·82 and 2·25 were estimated to correspond to $[Ca^{2+}]_i$ of 0 nM, 1000nM and 2000nM respectively.

RESULTS

1. Electrical field parameters and external Ca^{2+} control the rate of egg activation induced by electrostimulation

As a prelude to investigations on the intracellular changes induced by electroactivation, optimal electrical field parameters and pulsing media were determined. Successful egg activation was measured by the formation of morphologically normal

pronuclei by 15h post-stimulation; eggs with MII chromosomes at this stage were classified as non-activated.

Fig. 1 shows both the relationship between DC field strength and activation rates and the importance of the presence of extracellular Ca^{2+} in the pulsing medium on egg activation. It is apparent that for a given pulse duration (60 μs) and in the presence of extracellular Ca^{2+} in the pulsing medium activation rate increases with the elevation of the field strength (in the range of 0.3kV/cm to 1.0kV/cm). It is further clear that under our experimental conditions the optimal DC field strength for high activation rates coupled with a low incidence of egg lysis is between 1.0kV/cm and 1.5kV/cm. The results in Fig. 1 also show that some eggs exposed to a field strength below 0.5kV/cm are activated while the others remain at MII, illustrating that individual eggs require different threshold field strength to induce activation. On the other hand, the results in Fig. 1 demonstrate that despite adequate electrostimulation, treated eggs will fail to undergo activation in the absence of Ca^{2+} in the pulsing medium.

The effect of pulse duration on egg activation rate is summarized in Fig. 2. Firstly, there results show that using a single field strength (e.g 1.0kV/cm) and a standard pulsing medium the number of eggs which undergo activation increases with the increase of pulse duration (in the range of 10 μs to 80 μs). Secondly, the results demonstrate that high rates of activation are the product of an inverse relationship between DC field strength and pulse duration. Therefore, when the field strength is low (0.7kV/cm) a long pulse duration is required to induce activation, while at a high field strength (2.5kV/cm) a short pulse duration is sufficient to induce a high rate of activation, indicating that a delicate balance must exist between the pulse duration and the field strength.

In summary, the combined results demonstrate that, under adequate experimental

conditions eggs can be efficiently activated by electrical stimuli and that the rate of activation is primarily determined both by the electrical field parameters and the presence of extracellular Ca^{2+} in the pulsing medium.

2. Electrostimulation induces both cortical granule exocytosis and the reprogramming of protein synthesis in a manner analogous to that at fertilization

Cortical granule exocytosis. Cortical granules are membrane bound secretory granules located immediately beneath the cell membrane in the mammalian eggs. Typically, sperm penetration triggers the fusion of the cortical granule membrane with the resultant release of the granule's content. An ultrastructural analysis of the efficiency of electrostimulation for the induction of cortical granule exocytosis was undertaken by fixing eggs at various times post-stimulation.

Fig. 3 shows the ultrastructural changes which occur in eggs following stimulation using conditions which induce 100% egg activation. Fig. 3A shows the distribution, before stimulation, of numerous cortical granules which appear as membrane bound vesicles containing electron dense material and are located immediately beneath the plasma membrane. A sharp reduction in the number of cortical granules is apparent in groups of comparably treated eggs by 1 min post-stimulation (Fig. 3B) and by 10 min post-stimulation almost all the cortical granules are absent from just beneath the egg membranes (Fig. 3C). The ultra structural changes observed after electrostimulation both to the cortical granules and plasma membrane appear similar to those observed in porcine eggs after fertilization (see Cran & Cheng, 1986 for details).

Reprogramming of protein synthesis in activated eggs. To determine whether electrostimulation induces the same programme of protein reprogramming as that elicited by

sperm penetration comparisons were made between polypeptide profiles from unfertilized eggs, zygotes and parthenogenetically activated eggs. The results in Fig. 4 show that sperm penetration induces changes in the polypeptide profile which differ sharply from that of unfertilized eggs. There is, however, a close similarity confirmed by densitometry, between polypeptide profiles from zygotes and parthenogenetic eggs activated by electrostimulation.

In summary, the combined results of this series of experiments reveal that adequate electrostimulation causes porcine eggs to undergo both cortical granule exocytosis and protein reprogramming in a manner which mimics that induced by sperm during fertilization.

3. Egg activation induced by electrostimulation is attributed to a transient increase in $[Ca^{2+}]_i$ immediately following stimulation

The purpose of this series of experiments was to determine some of the extracellular factors involved in regulating $[Ca^{2+}]i$ changes and activation of eggs following electrostimulation.

Effect of extracellular Ca^{2+} on $[Ca^{2+}]_i$ levels following stimulation. Analysis of 80 eggs showed that before electrostimulation the basal $[Ca^{2+}]_i$ level is low (20-60 nM) and that the sharp rise after stimulation depends both on the presence of extracellular Ca^{2+} and on a Ca^{2+} influx. Thus, under the same electrical field conditions the $[Ca^{2+}]_i$ rise after stimulation increases in parallel with increases in the concentration of extracellular Ca^{2+} (Fig. 5). Secondly, despite adequate electrostimulus, eggs show no elevation of $[Ca^{2+}]i$ in a pulsing medium devoid of Ca^{2+}. Moreover, using standardised electrostimulation procedures all eggs stimulated in pulsing medium containing 100 μM Ca^{2+} were activated as judged by pronuclear formation whilst none of those stimulated in the absence of extracellular Ca^{2+} formed pronuclei. We suggest that the results of these different

experiments indicate (i) that the intracellular free Ca^{2+} rise after stimulation directly triggers the activation programme and (ii) that a Ca^{2+} influx immediately following stimulation plays an essential role in raising the $[Ca^{2+}]_i$ levels.

Effect of electrical field strength and pulse duration on $[Ca^{2+}]i$ changes in eggs. The results presented in Fig. 6 and 7 extend those presented earlier (Fig.s 1 and 2) by showing that field strength and pulse duration strongly affect not only activation rate but also $[Ca^{2+}]i$ in porcine eggs. Thus, in a standard pulsing medium the greater the field strength or the longer the pulse duration the higher the $[Ca^{2+}]_i$ in treated eggs. Interestingly, all eggs showed similar $[Ca^{2+}]_i$ changes under optimal stimulation conditions (100% activation). By contrast, marked individual variations in $[Ca^{2+}]_i$ were observed in eggs subjected to sub-optimal electrostimulation conditions (below 60% activation), thus, it was found that under the same stimulation condition some eggs have shown significant increase in $[Ca^{2+}]_i$ while the others showed very little response.

In summary, the results in this part of the investigation illustrate that $[Ca^{2+}]_i$ increase following electrostimulation is the direct trigger that initiates the activation programme and that the $[Ca^{2+}]_i$ changes is determined by both an influx of Ca^{2+} and the field parameters.

4. Electrostimulation is not able to reporduce the pattern of $[Ca^{2+}]_i$ changes initiated by sperm penetration

An important physiological characteristic of the way in which sperm activate eggs during hamster fertilisation is the induction of a series of repetitive transient rises in $[Ca^{2+}]_i$ that persist for more than an hour after penetration (Miyazaki, 1990). Our present results extend these observations both by studying calcium transients in the eggs of another species (pig) and by comparing the pattern and characteristics of the $[Ca^{2+}]_i$ changes in eggs after

fertilization and after electro activation.

To determine the $[Ca^{2+}]_i$ changes initiated by sperm penetration, different groups of the fura-2 prelabelled zona-free eggs were examined by dynamic imaging immediately post-insemination. The results show that the basal $[Ca^{2+}]_i$ level in these labelled eggs remained very low (at the range of 20 to 75 nM) during the first 4h post-insemination. Cytological assessment showed that no sperm had penetrated the eggs during this period of time. This observation is in agreement with the results of Cheng (1985) who reported that sperm require approximately 4h of preincubation before becoming capable of fertilising zona-free eggs. Indeed, the imaging studies from three experiments, consisting of 14 eggs, showed that Ca^{2+} oscillation were initiated in some of the eggs from 4h post-insemination whilst others were delayed for a further 0.5-1h before responding. The results in Fig. 8 show that sperm penetration induces a series of Ca^{2+} spikes in matured pig eggs. The oscillations in these fertilising eggs sometimes last for more than 3h. However, individual eggs showed difference in both the spiking frequency and amplitude. Characeristically, there was always a gradual increase in basal $[Ca^{2+}]_i$ level before each Ca^{2+} spike. It appears that the spike was triggered only when the basal $[Ca^{2+}]_i$ reached a threshold level. In most cases, the Ca^{2+} rise reached a peak in 10-15 seconds, remained at this high level for 6-10 seconds and then declined with the same dynamic as seen during the rise phase. Interestingly, it was found that if the basal $[Ca^{2+}]_i$ level in an egg was high then the threshold $[Ca^{2+}]_i$ required to initiate Ca^{2+} spiking was also high. Nevertheless, in the same egg during the early stage of oscillation the threshold required to induce spiking remained highly consistent. On the other hand, our preliminary results show that even in the same eggs (n=8) the pattern of Ca^{2+} oscillations in the later stages of fertilisation (approximately 2.5h post the initiation of Ca^{2+} oscillation) differed from that of the early stage (Fig. 9).

In contradiction to the spiking pattern observed after sperm penetration, Fig. 10 shows that a single electrical stimulus is only capable of eliciting a single Ca^{2+} transient in pig eggs. Further analysis revealed that the stimulation caused the eggs to increase their $[Ca^{2+}]_i$ levels from the resting (in the range of 20-50 nM) to a peak of 1.0-2.0 μM in 10-20 seconds which then declined slowly over several minutes (3-10 min); the precise decay time was dependent upon the DC field parameters and the extracellular Ca^{2+} concentration in the pulsing medium. Further Ca^{2+} transients do not occur in electrically activated eggs unless additional electrical pulses are administered. Moreover, the results showed that the $[Ca^{2+}]_i$ peaks induced by the same stimulation declined following repeated treatments, although the pattern of the Ca^{2+} rise remained similar. Imaging further revealed that the Ca^{2+} transient induced by electrostimulation travels in the form of a wave (Fig. 11) which is always initiated in the region adjacent to the positive electrode and then spreads over the entire eggs at a velocity of 5-10 μm/s. However, if more than two pulses are applied during a stimulation, a second Ca^{2+} wave is then also initiated adjacent to the negative electrode. The two oposing waves travel towards each other until they meet. Although Ca^{2+} waves have not previously been observed in pig eggs, Miyazaki (1986) has reported that in hamsters a Ca^{2+} wave begins near the site of sperm attachment, and then spread over the entire eggs within seconds.

In summary, the results of these experiments demonstrate that the pattern and characteristics of $[Ca^{2+}]_i$ transients induced by electrostimulation differs sharply from those observed in fertilising eggs. It is, therefore, concluded that the pattern of $[Ca^{2+}]_i$ changes initiated by sperm penetration can not be reproduced by electrostimulation.

The results of our experiments show firstly that pig eggs activated using optimal electrostimulation protocols not only complete meiosis but also undergo cortical granule exocytosis and protein reprogramming in a manner analogous to that observed after fertilization. Dynamic video imaging using Fura-2 as a Ca^{2+} probe has extended our results by showing that electrical stimulation activates porcine eggs by inducing a transient increase in intracellular free Ca^{2+}, the amplitude of which is determined by the concentration of extracellular Ca^{2+} and the electrical field strength and the pulse duration. In the absence of extracellular Ca^{2+}, despite adequate electrical stimulation, no transient increase in $[Ca^{2+}]_i$ has been observed in treated eggs and none have undergone activation, illustrating (1) that Ca^{2+} plays a central role in the activation and (2) that an influx of Ca^{2+} across the plasma membrane following stimulation is the direct trigger that initiates the intracellular Ca^{2+} changes.

The striking difference in intracellular Ca^{2+} characteristics between eggs activated by electrostimulation and those activated by sperm penetration represents the second important finding. Our results demonstrate clearly that a single electrical stimulus only induces a single transient $[Ca^{2+}]_i$ increase which shows a quick increase phase and a very slow recovery phase lasting for minutes; further transient increases are not observed unless additional stimuli are applied. Moreover, when multiple stimulations are given each resultant Ca^{2+} transient is similar to that which occurs during the first stimulation. By contrast during fertilization, the sperm induces multiple Ca^{2+} spikes of short duration and with a fairly regulate frequency. The entire Ca^{2+} oscillation activity induced by fertilization can persist for at least three hours following sperm-egg fusion. It therefore appears that although repeated electrical can induce multiple Ca^{2+} transients but with a pattern of Ca^{2+} oscillations

that differs markedly from that induced by sperm at fertilization. A comparison of our dynamic imaging with that of Miyazaki (1990) shows a number of interesting similarities and differences in the way in which the Ca^{2+} signal is propagated after sperm penetration as compared with electrostimulation. In fertilised hamster eggs the Ca^{2+} wave is initiated at the site of sperm-egg fusion and then spreads across the entire egg within seconds (Miyazaki *et al.*, 1986). Our results show that after a single electrical stimulus a Ca^{2+} wave is always initiated at a point adjacent to the positive electrode (Fig. 11); the Ca^{2+} wave spreads across the egg but more slowly than that induced by sperm fusion. The administration of multiple pulses during a stimulation can also initiate a second Ca^{2+} wave with an initiation site adjacent to the negative electrode, the two waves propagated from both directions and then converged.

On the basis of the differences in the pattern of Ca^{2+} transients and to a lesser extent on the mode of Ca^{2+} propagation we postulate that the mechanisms involved in egg activation by electrostimulation differ from those that operate during sperm-mediated activation. A major difficulty in testing this suggestion is that two fundamentally different ideas presently exist on the means by which sperm generate Ca^{2+} transients in eggs. On the one hand it is reported that sperm act by binding to external receptors that are linked to the egg's GTP-binding proteins, these in turn activate a phospholipase C that leads both to the generation of IP3, and the release of Ca^{2+} from intracellular stores (Jaffe *et al.*, 1988; Whitaker, 1989). The alternative hypothesis proposes that the sperm trigger Ca^{2+} release by first fusing with the eggs and then delivering a soluble factor into the egg cytoplasm (Dale, 1988; Swann, 1990). Berridge (1990) has extended this debate by reviewing in detail the different working models that seek to explain how a initial stimulus is thereafter transformed, firstly into a intracellular Ca^{2+} wave and secondly into the persistent series of

Ca^{2+} oscillations which characterised fertilization. Despite disagreements about the proposed models, it is generally accepted that inositol-trisphosphate (IP_3) plays a key role in regulating oscillatory activity in living cells. Berridge (1990) favours a model in which IP_3 acts as a second messenger to stimulate Ca^{2+} release from an IP_3-sensitive pool in the cells. He postulates further that this intracellular Ca^{2+} coupled with an influx of extracellular Ca^{2+} provides the constant flux Ca^{2+} required to prime and trigger the release, often in the form of a wave of an IP_3-insensitive Ca^{2+} store by Ca^{2+}-induced Ca^{2+} release. We believe that the absence of Ca^{2+} oscillations after electroactivation is a reflection of an entirely different form of intracellular signalling from that observed after fertilization. It appears probable but has not yet been definitively proven, that electrostimulation induces temporary pore formation in the egg membrane, an influx of extracellular Ca^{2+} via ion diffusion through the pores and a consequent single release of intracellular Ca^{2+} from IP_3-insensitive stores. Successive electrical pulses each induce temporary pore formation and a resultant single transient. It is still an open debate as to whether electrical stimulation can release Ca^{2+} from the IP_3-insensitive pool in the complete absence of any IP_3 involvement.

A number of other interesting cell biological observations have been made during the course of these experiments. (i) An inverse relationship has been established between field strength and pulse duration in determining the rate of egg activation. (ii) Marked heterogeneity in the responsiveness of eggs to electrical stimuli have been recorded; under the same electric field conditions some eggs will activate while others will not. (iii) The $[Ca^{2+}]_i$ level increases as the field strength or pulse duration is increased. A ready explanation for these observations can be provided by linking the electrofusion membrane pore hypothesis (Sun & Moor, 1989) with the need for a threshold $[Ca^{2+}]i$ level to initiate egg activation. Individual egg responsiveness could result from differences in either pore

formation or intracellular Ca^{2+} sensitivity. We have postulated that electrically induced pore formation results from the reorientation of charged molecules in the cell membrane; small differences in the distribution and nature of membrane proteins in the egg would be sufficient to induce significant variations in pore size even when the same electrical field strengths are applied. The entry of sufficient extracellular Ca^{2+} to reach the threshold required to activate the egg clearly relates both to pore development and extracellular Ca^{2+} concentrations. Thus, we suggest that with a given Ca^{2+} concentration in the medium, the overall $[Ca^{2+}]_i$ increase is primarily determined by the energy generated by the electrical field and that activation will occur in only those eggs whose $[Ca^{2+}]_i$ exceeds a trigger threshold. We suggest further that increasing the electrical energy by increasing either the field strength or pulse duration may not only increase the influx of extracellular Ca^{2+} which probably in turn induces a more complete release of Ca^{2+} from intracellular stores.

The role in development of the sequence of Ca^{2+} oscillations that occur in eggs after fertilization is still to be determined. However, our present findings show that a single Ca^{2+} transient is sufficient to induce both protein reprogramming and cortical granules exocytosis in a manner which appears to mimic precisely that induced by sperm at fertilization. It is therefore unlikely that multiple Ca^{2+} transients play a critically important role in these two biological processes. The experiments of Ozil (1990) suggest that Ca^{2+} oscillations following egg activation may well have important implications for preimplantation development. However, it is possible that the results observed by him were due primarily to either electrical field parameters or the $[Ca^{2+}]_i$ level changes following electrostimulation rather than to Ca^{2+} oscillations. It seems clear that much interesting research is likely to be done in the next few years on the causes and effects of Ca^{2+} oscillations in eggs and embryos. On the other hand, our observation on the inability of electro-activation in

initiating Ca^{2+} oscillation in the eggs following the stimulation has also raised our interest in the relationship between the developmental capacity of nuclear-transplanted eggs and the lack of appropriate calcium oscillations in these eggs following electrofusion. It seems that if calcium oscillations in the eggs are indeed essential for normal preimplantation development, the lack of this important biological process in the artificially activated eggs may well explain why the development of the nuclear-transplanted eggs obtained by electrofusion is generally very poor; it also means that the current electrofusion procedures used for nuclear transfer in the mammalian species are inadequate and should be supplemented with other approaches in order to mimic those normally initiated by sperm during fertilization.

Moreover, it is also evident from the present investigation that the $[Ca^{2+}]_i$ transient rise resulting from electrostimulation is the direct trigger of inducing egg activation : despite adequate electrostimulation, the treated eggs will not be activated in a pulsing medium devoid of Ca^{2+}. By applying this observation to nuclear transplantation studies, we have found that fusion between karyoplasts and MII cytoplasts can be efficiently achieved without inducing egg activation. This enables us, therefore, to develop a modified nuclear transplantation procedure in which donor nuclei can be conditioned for various periods of time, before initiation of egg activation by a second set of electrical stimuli in a pulsing medium containing Ca^{2+}. We postulate that such a modified procedure, by conditioning the donor nuclei in the MII cytoplasm for adequate periods of time, will enhance complete nuclear reprogramming and therefore improve the developmental capacity of the nuclear-transplanted eggs.

FIGURE LEGENDS

Fig. 1 Effect of DC field strength and extracellular Ca^{2+} on egg activation rates. The eggs were stimulated at different DC field strength with two pulses of 60 μs duration separated by 0.2 sec pulse interval either in the basic pulsing medium consisting of 0.3 M mannitol, 100 μM Ca^{2+}, 100 μM Mg^{2+} and 0.1% of PVA (●) or in Ca^{2+}-free pulsing medium (■). Each point represents 20 to 30 eggs.

Fig. 2 The relationship between DC pulse duration and field strength on the rate of activation. Eggs were stimulated with two pulses of varying pulse duration using weak (■, 0.7 kV/cm), medium (○, 1.0 kV/cm) and high (●, 2.5 kV/cm) external DC field strength separated by 0.2 sec pulse interval in the basic pulsing medium.

Fig. 3 Electron micrographs of porcine eggs showing (A) the presence of cortical granules (CG) in control eggs, (B) CG exocytosis at 1 min post-electrostimulation, and (C) complete release of CGs at 10 min post-stimulation. The eggs in B and C were stimulated at 1.5 kV/cm, two pulses of 60 μs duration in the basic pulsing medium.

Fig. 4 Fluorgraph of SDS-PAGE gel showing polypeptides synthesised by porcine MII control eggs (1-2), parthenogenetically activated eggs (3-4) and normally fertilised zygotes (5-6). At 45h of maturation, the MII eggs were either treated by electrostimulation or inseminated by sperm, the control eggs were cultured without any further treatment. After stimulation for 15h or inseminated for 21h, both the eggs and zygotes were labelled with [^{35}S] methionine for 3h, and then run on 8-15% SDS gradient gels. The detectable differences among them in polypeptide compositions are indicated by arrows. Each track in these groups represented 5 eggs or zygotes.

Fig. 5 The effect of extracellular Ca^{2+} concentrations on $[Ca^{2+}]_i$ changes in electrosimulated eggs. Following intensive washing in a pulsing medium, Fura-2 labelled eggs were transferred to the stimulation chamber and their basal $[Ca^{2+}]_i$ levels were recorded. Thereafter, the eggs were electrically activated and the $[Ca^{2+}]_i$ changes were measured. All the eggs in this study were treated under the same electrical field conditions (1.0 kV.cm, two pulses of 60 μs duration separated by 0.2 sec interval). The pulsing media had common basic components (0.3 M mannitol, 100 μM Mg^{2+}, and 0.1% PVA), but differed in their Ca^{2+} concentrations. Each bar represents the mean $\pm$ SEM of the maximum $[Ca^{2+}]_i$ levels detected in three groups of eggs (n=15-20).

Fig. 6 Effect of DC field strength on $[Ca^{2+}]_i$ in porcine eggs. Each group of eggs was stimulated at a defined DC field strength with two pulses of 60 μs duration in the basic pulsing medium containing 100 μM Ca^{2+}. Each bar represents the mean $\pm$ SEM of the maximum $[Ca^{2+}]_i$ levels of the treated eggs (n=12-18).

Fig. 7 Effect of DC pulse duration on $[Ca^{2+}]_i$ changes in eggs following stimulation in a basic pulsing medium containing 100 μM Ca^{2+}. The eggs were stimulated with two pulses of varying pulse duration using a field strength of 1.0 kV/cm. Each bar represents the mean + SEM of the maximum $[Ca^{2+}]_i$ levels in three groups of eggs (n=12-18).

Fig. 8 Pattern of Ca^{2+} transient rises observed in porcine eggs activated by sperm penetration. A and B show two different fertilised eggs which were oscillating at different spiking frequencies and amplitudes.

Fig. 9 An example of Ca^{2+} oscillation patterns of the same egg at different times after fertilization (A, within 1.5 hour of sperm penetration; B, 3.0-3.2h after sperm penetration).

Fig. 10 A typical example of the pattern of Ca^{2+} transient increase in porcine eggs induced by a single electrostimulation (A) and by multiple ones (B). The eggs were stimulated at 1.0 kV/cm with two pulses of 60 μs duration in the basic pulsing medium. Detection of the $[Ca^{2+}]_i$ changes following the stimulation(s) was also performed in the same pulsing medium at room temperature.

Fig. 11 Propagation of a Ca^{2+} transient in a porcine egg following electrostimulation. The egg was stimulated by a single pulse of 60 μs duration at the field strength of 1.0 kV/cm in the basic pulsing medium. The stimulation was given at the second frame. The frame interval is 1.8 second.

Claims

1. A process for nuclear transplantation comprising the steps of:

a) preparing a recipient cytoplast wherein the cytoplast is from an oocyte at the metaphase II stage of meiosis (MII oocyte);
b) preparing a donor nucleus; and
c) fusing the nucleus and cytoplast to produce a reconstituted embryo;

the process being carried out in vitro in a suspension medium;

characterised in that the suspension medium is a physiologically acceptable aqueous solution which contains substantially no free calcium ions.

2. A process for nuclear transplantation wherein a donor nucleus and a recepient cytoplast are fused in suspension in an aqueous medium under conditions wherein the medium contains substantially no extracellular free calcium ions, whereby the fusion occurs without inducing activation of the resultant reconstituted embryo.

3. A process for nuclear transplantation comprising the steps of:

i) carrying out the process of any preceding claim;

ii) maintaining the resultant reconstituted embryo without activation for a selected period of time so as to allow nuclear-cytoplasmic conditioning to occur in the intracellular environment, and;

iii) stimulating the reconstituted embryo to cause activation of developmental processes.

4. A process according to any preceding claim, in which the suspension medium contains less than 100μM Ca^{2+}.

5. A process according to any preceding claim, in which the suspension medium contains less than 10μM Ca^{2+}.

6. A process according to any preceding claim, in which the suspension medium contains no free calcium ions.

7. A process according to any preceding claim, in which the medium contains about 0.3M Mannitol, about 100μM Mg^{2+} in double distilled water at a pH of about 6.8 to about 7.0.

8. A process according to any preceding claim, in which fusion is carried out by electrofusion.

9. A process according to claim 8, in which electrofusion is achieved using a pulse which provides an electric field of about 0.5-2.0 KV/cm preferably 1.0-1.5 KV/cm in the time period range 0.10-100μs preferably 10-80μs.

10. A process according to any of claims 1 to 7, in which fusion is carried out by the use of Sendai virus.

11. A process according to any of claims 1 to 7, in which fusion is carried out by the use of an added solution of polyethylene glycol.

12. A process according to claim 3, wherein the selected period of time during which the reconstituted embryo is maintained without activation is up to several days.

13. A process according to claim 3, wherein the selected period of time during which the reconstituted embryo is maintained without activation is 2-24 hours.

14. A process according to claim 3, wherein during said selected period the reconstituted embryo is frozen, cryogenically stored, and subsequently thawed prior to activation.

15. A process according to claim 3, wherein activation is carried out by stimulation with an electric pulse, in aqueous medium in the presence of extracellular free calcium ions, preferably at a concentration of 10-100μM.

16. A process according to claim 3, wherein activation is carried out by stimulation with multiple transient electric pulses.

17. A process according to claim 3, wherein activation is carried out by stimulation with a chemical activator.

18. A process according to claim 17, wherein the chemical activator is selected from Ethanol, Thimerosol and IP3.

19. A process according to claim 3, wherein activation is carried out by a biological activator.

20. A process according to claim 19, wherein the biological activator is sperm.

21. A process according to claim 20, wherein the sperm has been treated by irradiation so as to inactivate the sperm DNA.

22. A process according to claim 16, wherein the multiple transient electric pulses cause intracellular calcium ion transient increases which approximate to those produced by sperm during fertilisation of an embryo.

23. A process for nuclear transplantation to achieve fusion of a donor nucleus and a recipient cytoplast from an MII oocyte without inducing embryo activation, substantially as herein before described.

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Documents considered relevant following a search in respect of claims

Category (see over)	Identity of document and relevant passages	Relevant to claim(s)
A	THERIOGENOLOGY 1990, 33(1), 301 - Behaviour of pig blastomare nuclei introduced into invitro matured ...	2
A	CELL DIFFERENTIATION 1984, 14(1), 47-52 - The fusion of oocytes of the starfish Aphelasterias japonica III Reconstruction ...	2

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Category	Identity of document and relevant passages	Relevant to claim(s)

Categories of documents

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